

ORIGINAL ARTICLE

Selection of a highly invasive neuroblastoma cell population through long-term human cytomegalovirus infection

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The human cytomegalovirus (HCMV) is suspected to increase tumour malignancy by infection of cancer and/or stroma cells (oncomodulation). So far, oncomodulatory mechanisms have been attributed to the presence of HCMV and direct action of its gene products on cancer cells. Here, we investigated whether the prolonged presence of HCMV can result in the irreversible selection of a cancer cell population with increased malignancy. The neuroblastoma cell line UKF-NB-4 was long-term (200 passages) infected with the HCMV strain Hi91 (UKF-NB-4^{Hi}) before virus eradication using ganciclovir (UKF-NB-4^{HiGCV}). Global gene expression profiling of UKF-NB-4, UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells and subsequent bioinformatic signal transduction pathway analysis revealed clear differences between UKF-NB-4 and UKF-NB-4^{Hi}, as well as between UKF-NB-4 and UKF-NB-4^{HiGCV} cells, but only minor differences between UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells. Investigation of the expression of a subset of five genes in different chronically HCMV-infected cell lines before and after virus eradication suggested that long-term HCMV infection reproducibly causes specific changes. Array comparative genomic hybridisation showed virtually the same genomic differences for the comparisons UKF-NB-4^{Hi}/UKF-NB-4 and UKF-NB-4^{HiGCV}/UKF-NB-4. UKF-NB-4^{Hi} cells are characterised by an increased invasive potential compared with UKF-NB-4 cells. This phenotype was completely retained in UKF-NB-4^{HiGCV} cells. Moreover, there was a substantial overlap in the signal transduction pathways that differed significantly between UKF-NB-4^{Hi}/UKF-NB-4^{HiGCV} and UKF-NB-4 cells and those differentially regulated between tumour tissues from neuroblastoma patients with favourable or poor outcome. In conclusion, we present the first experimental evidence that long-term HCMV infection can result in the selection of tumour cell populations with enhanced malignancy.

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INTRODUCTION

Human cytomegalovirus (HCMV), a ubiquitous herpes virus, infection leads (in immunocompetent individuals usually symptomless) to a life-long persistence after primary infection. About 50 to 100% of the general adult population is infected. HCMV routinely reactivates, but this is usually controlled by the host immune response in healthy people. In immuno-compromised individuals such as recipients of organ transplants or AIDS patients, HCMV is a major pathogen.^{1,2}

HCMV has been suspected to have a role in cancer diseases for decades, based on seroepidemiological evidence and on the detection of viral DNA, messenger RNA (mRNA) and/or antigens in tumour tissues.^{2,3} However, in contrast to viruses from different RNA virus (retrovirus, flavivirus) and DNA virus (hepadna virus, herpes virus, papova virus) families that are known to be oncogenic in humans,⁴ HCMV is not considered a tumour virus because of a lack of proven transformation potential in human cells.² With our studies about the influence of HCMV on cancer cells,^{5–7} we introduced the concept of oncomodulation, meaning that HCMV may infect cancer cells and/or stromal cells in established tumours and increase

tumour malignancy also in the absence of transformation potential.^{1–3,6,8,9} In the meantime, oncomodulatory effects exerted by HCMV or single cytomegalovirus proteins have been reported *in vitro* and *in vivo* by numerous groups.^{2,3,8–11} Moreover, application of sensitive (although not yet indisputably accepted) pathological methods applied by numerous independent research groups indicated the presence of HCMV and/or virus constituents in cancers from different cancer entities.^{1–3,12–17} In glioblastomas, the presence of HCMV was correlated with higher disease stage and worse outcome.^{1–3,12,18,19} In addition, expression of HCMV proteins appeared to promote oncogenic signalling events.^{2,3,12,20,21}

Neuroblastoma, a paediatric cancer entity, has been associated with increased HCMV antibody titres and HCMV immediate-early antigen (IEA) expression in a fraction of tumours.^{2,3,22,23} After primary HCMV infection of different neuroblastoma cell lines, a balance is established between virus production and cell division.^{6,7,24,25} Chronically HCMV-infected neuroblastoma cells show a more malignant phenotype indicated by properties such as increased invasive potential, metastasis formation in nude mice and resistance to chemotherapy.^{2,6,7,24}

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So far, HCMV-induced oncomodulatory effects were attributed to the presence of HCMV and direct action of its gene products,^{2,3,10,24} and therefore suspected to be reversible after virus eradication. Here, we investigated the effects of long-term HCMV strain Hi91 infection on UKF-NB-4 neuroblastoma cells. Long-term HCMV-infected (UKF-NB-4^{Hi}) cells showed a very close relationship with ganciclovir-cured UKF-NB-4^{Hi} (UKF-NB-4^{HiGCV}) cells at the level of gene expression and genomic copy number changes, whereas substantial differences were detected between UKF-NB-4^{Hi}/UKF-NB-4^{HiGCV} cells and parental UKF-NB-4 cells. Moreover, UKF-NB-4^{HiGCV} showed the same increased invasive potential as UKF-NB-4^{Hi} cells compared with UKF-NB-4. Bioinformatics signal transduction pathway analysis suggested a substantial overlap in pathways differentially regulated between UKF-NB-4^{Hi}/UKF-NB-4^{HiGCV} cells and UKF-NB-4 cells, as well as between tumour tissues from neuroblastoma patients with poor or favourable outcome. These data indicate that the long-term presence of HCMV can result in the irreversible selection of a cancer cell population with increased malignancy. Investigation of the expression of a subset of five genes in additional long-term HCMV-infected neuroblastoma cells and their cidofovir- or ganciclovir-cured sub-lines suggested that long-term HCMV infection of different neuroblastoma cells reproducibly results in characteristic changes.

RESULTS

Establishment of chronically HCMV-infected neuroblastoma cells and virus eradication

UKF-NB-4 cells, derived from bone marrow metastases of a patient harbouring a MYCN-amplified stage IV neuroblastoma,²⁶ were infected once with the HCMV strain Hi91²⁷ at MOI 10 and then subcultured without further addition of virus (UKF-NB-4^{Hi}). Non-infected UKF-NB-4 cells were passaged in parallel as control. After primary infection, about 80% of UKF-NB-4 cells were HCMV infected (Figure 1). Five days after infection, the amount of viable cells was about 20%, as indicated by trypan blue staining (Supplementary Figure 1). After 200 passages, HCMV IEA and late

antigen expression remained detectable in UKF-NB-4^{Hi} cells, resulting in about 30–60% infected cells (Figure 1). Trypan blue staining indicated 70–80% viable cells (5 days after passaging of cells) (Supplementary Figure 1). Virus titres were 8.0×10^2 TCID₅₀ (tissue culture infectious dose)/ml at passage 1 (determined 5 days after primary infection), 4.5×10^2 TCID₅₀/ml at passage 100 and 1.4×10^3 TCID₅₀/ml at passage 200 (both detected 5 days after passaging). HCMV DNA copy numbers were $6.3 \times 10^5/10^6$ cells at passage 1 (determined 5 days after primary infection), $9.8 \times 10^4/10^6$ cells at passage 100 and $9.1 \times 10^5/10^6$ cells at passage 200 (both detected 5 days after passaging).

Thereafter, UKF-NB-4^{Hi} cells were treated with 20 μ M ganciclovir (a concentration that did not affect the viability of UKF-NB-4 or UKF-NB-4^{Hi} cells) for six passages until no HCMV gene expression was detected anymore (UKF-NB-4^{HiGCV}; Figure 1). Further cultivation of UKF-NB-4^{HiGCV} cells for 10 passages did not result in recurrence of HCMV gene expression (Figure 1). The absence of HCMV from UKF-NB-4^{HiGCV} cells was confirmed by virus yield assay and quantitative PCR detecting HCMV genome (Supplementary Figure 2).

Comparison of global gene expression in UKF-NB-4, UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells

Triplicates of UKF-NB-4, UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells were analysed for global cellular gene expression at the mRNA level using gene microarray (Affymetrix HGU133plus2, Santa Clara, CA, USA). Correlation analysis revealed a very high similarity between gene expression profiles in UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells but clear difference between these two cell lines and UKF-NB-4 (Figure 2a). More than 8000 genes were significantly differentially expressed (false discovery rate (FDR) < 0.05, corrected for multiple testing) between UKF-NB-4^{Hi} and UKF-NB-4 cells or UKF-NB-4^{HiGCV} and UKF-NB-4 cells, whereas only about 300 genes were differentially expressed between UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells (Figure 2b, Supplementary Table 1). Further analysis showed that nearly 90% of the genes significantly differentially expressed between UKF-NB-4^{Hi} and UKF-NB-4 cells and between UKF-

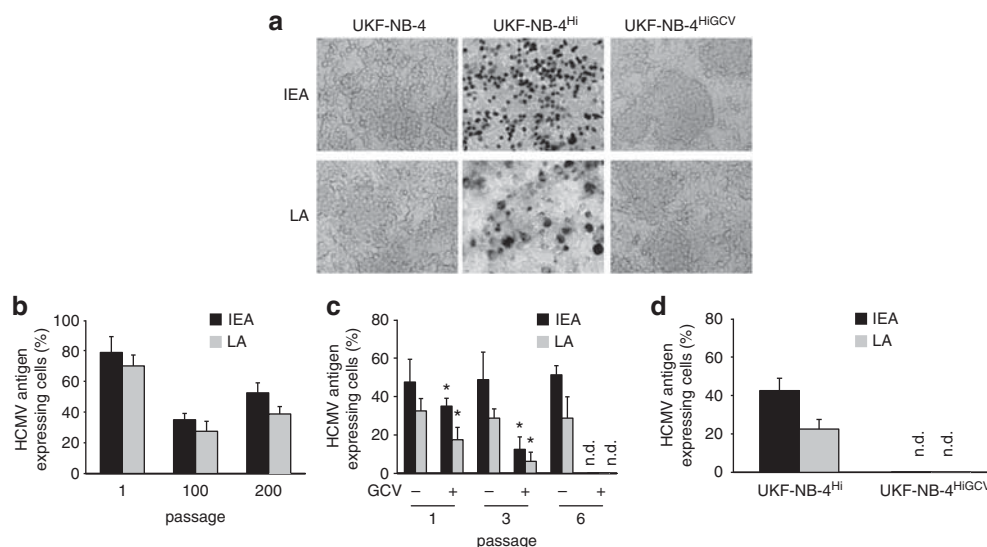


Figure 1. Establishment of UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells. UKF-NB-4 cells were infected once with HCMV strain Hi at MOI 10 and then subcultured without further addition of virus (UKF-NB-4^{Hi}). After 200 passages, UKF-NB-4^{Hi} cells were treated for six passages with 20 μ M ganciclovir (GCV) until no HCMV IEA or late antigen (LA) expression were detectable anymore. (a) Representative pictures showing immunostaining of UKF-NB-4, UKF-NB-4^{Hi} or UKF-NB-4^{HiGCV} cells for IEA or LA. (b) Fraction of HCMV antigen-expressing cells in UKF-NB-4^{Hi} cells at different passages after initial infection. (c) HCMV antigen expression in GCV-treated UKF-NB-4^{Hi} cells at different passages in comparison with non-treated UKF-NB-4^{Hi} cells. Values represent mean \pm s.d. from three independent experiments. * P < 0.05 relative to non-treated UKF-NB-4^{Hi} cells. (d) HCMV antigen expression in GCV-cured UKF-NB-4^{Hi} (UKF-NB-4^{HiGCV}) cells after cultivation for 10 passages in the absence of GCV in comparison with non-treated UKF-NB-4^{Hi} cells. n.d., not detectable.

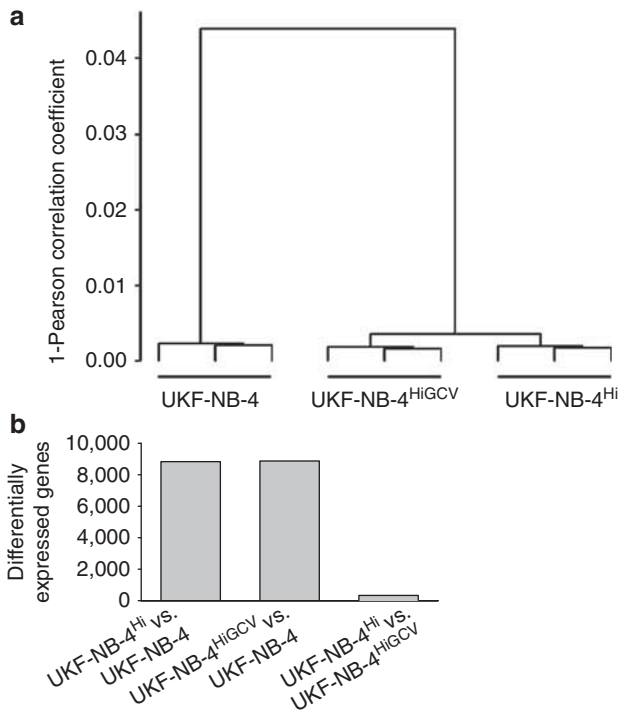


Figure 2. Comparison of global gene expression in UKF-NB-4, UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells by gene microarray. (a) Hierarchical cluster analysis based on the Pearson correlation coefficient. (b) Numbers of genes significantly differentially expressed (FDR < 0.05) between the investigated cell lines.

NB-4^{HiGCV} and UKF-NB-4 cells were changed consistently in both UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells (that is, were both over-expressed or both were underexpressed compared with UKF-NB-4; Supplementary Table 2). These data indicate that both UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} strongly differ from UKF-NB-4 cells by means of gene expression, whereas gene expression signatures of UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} were very similar.

Correlation analysis of UKF-NB-4 cells stored at the beginning of the experiments in parallel with primary infection (UKF-NB-4⁰) and UKF-NB-4 cells that had been cultivated in parallel with the UKF-NB-4^{Hi} cells for 200 passages (UKF-NB-4²⁰⁰) indicated a close relationship between these two passages of the UKF-NB-4 cell line but a substantial difference to UKF-NB-4^{Hi} cells (Supplementary Figure 3).

Bioinformatic signal transduction pathway analysis of global gene expression data

Bioinformatic signal transduction pathway analysis, performed using the PANTHER database (www.pantherdb.org), showed that the top five differentially regulated pathways for the comparisons UKF-NB-4^{Hi} vs UKF-NB-4 and UKF-NB-4^{HiGCV} vs UKF-NB-4 were the same, although the order differed (Table 1). In total, 13 pathways were significantly differentially regulated in both comparisons (P -value < 0.05, corrected for multiple testing; Table 1), whereas only three pathways were significantly differentially regulated between UKF-NB-4^{Hi} and UKF-NB-4 cells but not between UKF-NB-4^{HiGCV} and UKF-NB-4 cells (Supplementary Table 3). Four pathways were significantly differentially regulated between UKF-NB-4^{HiGCV} and UKF-NB-4 cells but not between UKF-NB-4^{Hi} and UKF-NB-4 cells (Supplementary Table 4). No significant differentially regulated pathways were detected between UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV},

confirming the high similarity of these cells at the level of gene expression.

Gross genomic differences of UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells compared with UKF-NB-4 cells by array comparative genomic hybridization (CGH)

Array CGH is a technology developed to detect genomic copy number variations. UKF-NB-4 cells were compared with UKF-NB-4^{Hi} cells or UKF-NB-4^{HiGCV} cells by array CGH in order to investigate whether genomic differences underlie the observed changes in gene expression. Results revealed virtually identical array CGH profiles in DNA copy number for both the comparisons UKF-NB-4^{Hi} vs UKF-NB-4 and UKF-NB-4^{HiGCV} vs UKF-NB-4 (Figure 3). There is evidence of aneuploidy, chromosome segment gain and loss and individual loci loss/gain. Specifically, we detected aneuploidy (gain) of chromosomes 6, 13 and 20, with loss of 14, 15 and 18. Structural gains were observed at terminal 2p, the whole of 9q (with a small portion of 9p), 12q, terminal 17p and 20q. Losses are detected of chromosome 1q (and about a third of proximal 1p), parts of chromosome 3, terminal 5q, a central portion of 8q, most of terminal 9p, terminal 15q (in addition to the aforementioned aneuploidy) and 20p. Reciprocal gains and losses of p and q arms on chromosomes 9 and 20 (and possibly chromosome 12) may indicate the presence of isochromosomes. Single locus gains (gene amplifications) were apparent on chromosomes 4q and 15q, with losses on 7p, 7q, 9p, 10 (near the centromere), 15q and 16q.

UKF-NB-4, UKF-NB-4^{Hi} or UKF-NB-4^{HiGCV} adhesion to and transmigration through endothelial monolayers

UKF-NB-4, UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} were investigated for cancer cell adhesion to and migration through human umbilical vein endothelial cell (HUVEC) monolayers. These events are considered to be important during metastasis formation. HCMV-infected neuroblastoma cells had been shown earlier to exhibit a stronger invasive potential than non-infected cells.²⁸ UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells both showed a strongly increased adhesion to endothelial cells (Figure 4a), as well as enhanced numbers of transmigrating cells (Figure 4b) when compared with UKF-NB-4. Moreover, UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells (but not UKF-NB-4 cells) disrupted endothelial cell monolayer integrity, resulting in focal plaques (that is, round openings that are not associated with endothelial cell HCMV infection or death as described²⁸) (Figure 4c). Previous results had shown that interaction of UKF-NB-4 cells with endothelial cells depended on $\alpha 5\beta 1$ integrin binding.²⁸ Here, adhesion of UKF-NB-4, UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells to and transmigration through endothelial cell monolayers could be inhibited by an antibody blocking $\alpha 5\beta 1$ integrin (Figures 4a and b). Moreover, antibody-mediated inhibition of $\alpha 5\beta 1$ integrin binding also interfered with endothelial cell monolayer disruption by UKF-NB-4^{Hi} or UKF-NB-4^{HiGCV} cells.

These findings suggest that the enhanced malignant properties of UKF-NB-4^{Hi} cells are at least partly retained in UKF-NB-4^{HiGCV} cells after and despite virus eradication.

Signal transduction pathways differentially regulated between UKF-NB-4 and UKF-NB-4^{Hi}/UKF-NB-4^{HiGCV} cells and between tumours from neuroblastoma patients with favourable or non-favourable outcome.

Next, signal transduction pathways differentially regulated between UKF-NB-4 and UKF-NB-4^{Hi}/UKF-NB-4^{HiGCV} cells were analysed in the context of two gene expression data sets derived from neuroblastoma patients. The first data set (Oberthuer *et al.*) was derived from a study comparing gene expression signatures between N-myc-amplified neuroblastoma tissues from patients with favourable outcome (event-free survival) or poor outcome (death or relapse of disease) (www.ebi.ac.uk/arrayexpress; accession E-TABM-38; Oberthuer *et al.*²⁹). The second data set (Asgharzadeh *et al.*) investigated gene expression in non-N-myc-

Table 1. Signal transduction pathways that are significantly differentially regulated ($P < 0.05$, corrected for multiple testing) in UKF-NB-4^{Hi} vs UKF-NB-4 and UKF-NB-4^{HiGCV} vs UKF-NB-4 cells as identified by PANTHER pathway analysis

Pathway (number of genes annotated in the pathway)	UKF-NB-4 ^{Hi} vs UKF-NB-4 (rank ^a /P-value/genes affected ^b)	UKF-NB-4 ^{HiGCV} vs UKF-NB-4 (rank ^a /P-value/genes affected ^b)
Wnt signalling (348)	1/1.76 × 10 ⁻¹⁰ /191	1/3.32 × 10 ⁻¹⁰ /190
Ras pathway (91)	2/1.57 × 10 ⁻⁶ /64	3/8.14 × 10 ⁻⁶ /62
Ubiquitin proteasome pathway (89)	3/3.69 × 10 ⁻⁶ /62	4/8.48 × 10 ⁻⁶ /61
Cadherin signalling pathway (168)	4/1.12 × 10 ⁻⁵ /96	2/6.21 × 10 ⁻⁶ /97
Parkinson disease (106)	5/7.04 × 10 ⁻⁵ /66	5/1.72 × 10 ⁻⁵ /68
Angiogenesis (229)	6/3.38 × 10 ⁻⁴ /115	12/8.50 × 10 ⁻³ /108
Integrin signalling pathway (227)	7/6.04 × 10 ⁻⁴ /113	9/2.51 × 10 ⁻³ /110
EGF receptor signalling pathway (150)	8/8.68 × 10 ⁻⁴ /81	6/8.91 × 10 ⁻⁴ /81
PDGF signalling pathway (187)	9/8.69 × 10 ⁻⁴ /97	11/6.33 × 10 ⁻³ /93
p53 pathway (136)	11/2.94 × 10 ⁻³ /73	7/9.63 × 10 ⁻⁴ /75
Huntington disease (172)	12/1.57 × 10 ⁻³ /87	8/4.23 × 10 ⁻³ /89
p53 pathways feedback loops 2 (66)	13/1.02 × 10 ⁻² /41	13/1.00 × 10 ⁻² /41
Cytoskeletal regulation by Rho GTPase (111)	16/3.93 × 1 ⁻² /58	16/3.86 × 10 ⁻² /58

Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor. ^aPathways were ranked by their P -values with the lowest P -value being rank 1. ^bPathway genes differentially expressed between the data sets (FDR < 0.05). In total, 25 431 genes were annotated in the PANTHER reference list. 8832 genes were differentially expressed between UKF-NB-4^{Hi} and UKF-NB-4, 8873 between UKF-NB-4^{HiGCV} and UKF-NB-4.

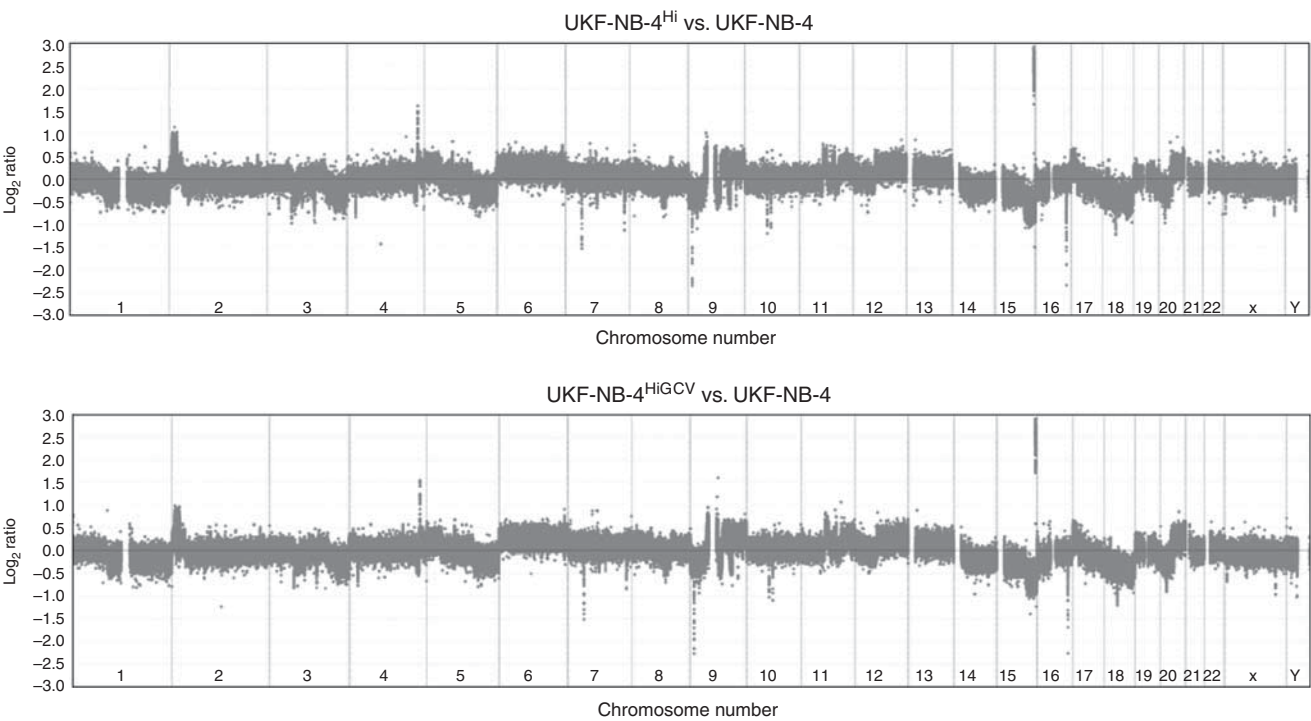


Figure 3. Array CGH of all 24 human chromosomes resulting from comparisons of UKF-NB-4^{Hi} cells (upper plot) and UKF-NB-4^{HiGCV} cells (bottom plot), both compared with UKF-NB-4 cells. Results suggest that both UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} have very similar karyotypes: whole-chromosome gain and loss (e.g., on chromosomes 6 and 18), losses and gains of chromosome segments, e.g., terminal 2p and 12q, as well as amplification and loss on individual loci.

amplified neuroblastoma tissues from patients with favourable outcome (no relapse) or poor outcome (relapse of disease) (www.ncbi.nlm.nih.gov/geo/; accession number GSE3446³⁰). In the Oberthuer *et al.* data set, 21 out of the 153 PANTHER pathways were significantly differentially regulated ($P < 0.05$, corrected for multiple testing) between favourable outcome and poor outcome patients. Nine of the 13 pathways that had been found to be differentially regulated between UKF-NB-4 and UKF-NB-4^{Hi}/UKF-NB-4^{HiGCV} were also significantly differentially regulated between favourable and poor outcome samples in

this data set (Table 2). In the Asgharzadeh *et al.* data set, 32 out of the 153 pathways were significantly differentially regulated, and 12 of the 13 pathways differentially regulated between UKF-NB-4 and UKF-NB-4^{Hi}/UKF-NB-4^{HiGCV} were also significantly differentially regulated between favourable and poor outcome patients (Table 2).

Moreover, 7 of the 13 pathways (Angiogenesis, platelet-derived growth factor signalling pathway, Ras pathway, p53 pathway, p53 pathway feedback loops 2, Parkinson disease, Integrin signalling pathway) belonged to the 10 most strongly differentially

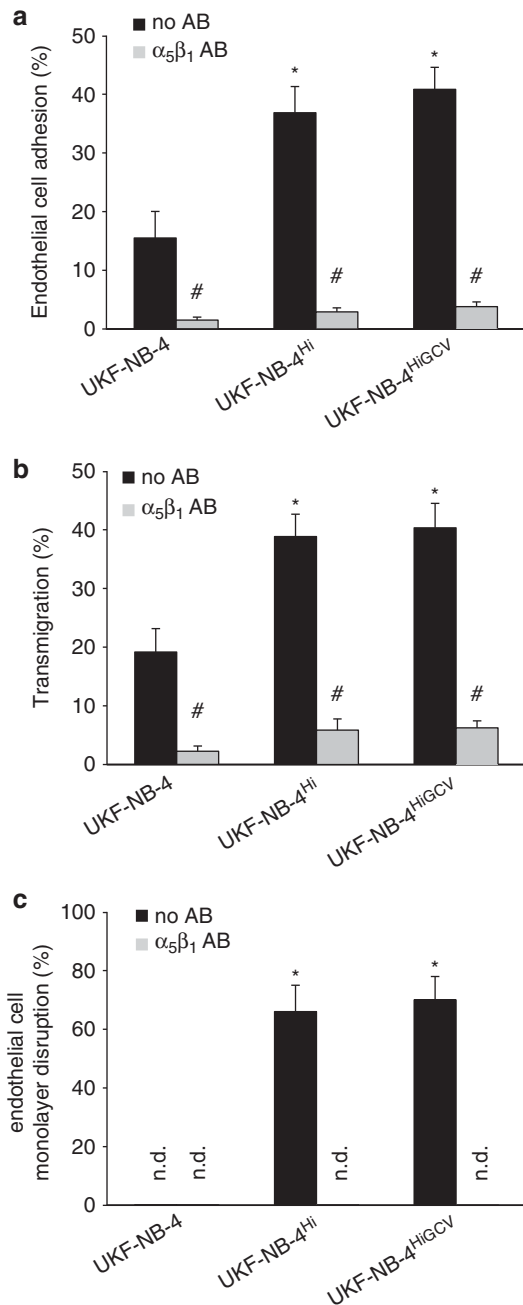


Figure 4. Adhesion of UKF-NB-4, UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells to endothelial cell monolayers, transmigration and endothelial cell monolayer disruption. **(a)** Fractions of cells that adhered to HUVEC monolayers (in the presence or absence of an $\alpha_5\beta_1$ -blocking antibody **(a, b)**) were expressed as the percentage of the total number of input cells. **(b)** Fractions of cells that transmigrated through HUVEC monolayers (in the presence or absence of an $\alpha_5\beta_1$ -blocking antibody **(a, b)**) were expressed as the percentage of the total number of adherent cells. **(c)** Focal endothelial cell monolayer disruption (in the presence or absence of an $\alpha_5\beta_1$ -blocking antibody **(a, b)**) was expressed as the percentage of the cell-free area. Values represent mean \pm s.d. from three independent experiments. * $P < 0.05$ relative to UKF-NB-4; # $P < 0.05$ relative to the corresponding cell line in the absence of $\alpha_5\beta_1$ -blocking antibody; n.d., not detectable; AB, antibody.

regulated pathways of the Oberthuer *et al.* data set. Six of the 13 pathways (Wnt signalling pathway, Angiogenesis, Huntington disease, platelet-derived growth factor signalling pathway,

epidermal growth factor receptor signalling pathway, p53 pathway) belonged to the 10 most strongly differentially regulated pathways of the Asgharzadeh *et al.* data set.

DISCUSSION

Oncomodulation is a concept postulating that HCMV may infect cancer cells and/or stromal cells in established tumours and increase tumour malignancy also in the absence or independently of transformation potential.^{2,3,6} To study long-term effects of HCMV on cancer cells, chronically HCMV-infected neuroblastoma cell cultures were established.^{6,7,24,25} Here, we show that long-term HCMV infection of UKF-NB-4 neuroblastoma cells results in the establishment of a cell population that is clearly distinct from the parental UKF-NB-4 cell line regarding global gene expression and genomic imbalances. Eradication of HCMV from chronically HCMV strain Hi91-infected UKF-NB-4 (UKF-NB-4^{Hi}) cells causes only minor effects on gene expression in the resulting UKF-NB-4^{HiGCV} cell line as indicated by comparison of differentially expressed genes, as well as by bioinformatics signal transduction pathway analyses. Genomic imbalances are virtually the same between UKF-NB-4^{Hi} and UKF-NB-4 cells and between UKF-NB-4^{HiGCV} and UKF-NB-4 cells as indicated by array CGH.

UKF-NB-4^{Hi} cells show an increased invasive phenotype indicating enhanced tumour cell malignancy compared with UKF-NB-4 cells. This more invasive phenotype is completely sustained in ganciclovir-cured UKF-NB-4^{HiGCV} cells. Thirteen signal transduction pathways are significantly differentially regulated between UKF-NB-4 cells and both UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells. The majority of these pathways are also significantly differentially regulated between tissues from neuroblastoma patients with favourable outcome and those from patients with unfavourable disease course (9 out of 13 in the Oberthuer *et al.* data set,²⁹ and 12 out of 13 in the Asgharzadeh *et al.* data set³⁰). This demonstrates that long-term HCMV infection of neuroblastoma cells can result in the establishment of a novel cell population with different malignant properties compared with the parental cell line. Moreover, malignant features, at least in part, no longer depend on the presence of HCMV or its gene products after long-term HCMV infection.

To enhance the confidence that the observed effects are associated with the presence of HCMV and to receive some more information about the specificity of the HCMV-induced selection process, five genes were selected that had been shown earlier to be significantly differentially expressed in various chronically HCMV-infected neuroblastoma cell lines relative to the corresponding non-infected cell lines: DHFR (encoding for dihydrofolate reductase), ENPP2 (encoding for autotaxin), KIAA0101 (encoding for a protein that is also known as PCNA-associated factor/p15(PAF)), NDRG1 (encoding for N-myc downstream regulated 1) and TMPRSS15 (also known as PRSS7, encoding for a protein known as enterokinase or transmembrane protease, serine 15).²⁵ These genes were also significantly differentially regulated between UKF-NB-4 and both UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells (as indicated by gene expression microarray data and confirmed by real-time PCR, Supplementary Table 5, Supplementary Figure 4), and between MHH-NB-11 cells chronically infected with HCMV strain AD169 (MHH-NB-11^{AD169},²⁵) and its sub-lines in which the virus was eradicated by the addition of ganciclovir (MHH-NB-11^{AD169GCV}) or cidofovir (MHH-NB-11^{AD169CDF}) compared with parental MHH-NB-11 cells (Supplementary Figure 5).

Acute infection of UKF-NB-4 cells with HCMV strain Hi91 did not affect the expression of DHFR, ENPP2, KIAA0101, NDRG1 or TMPRSS15. Moreover, chronic infection of UKF-NB-4 cells with varicella zoster virus strains did not influence the expression of these genes (data not shown), indicating that the expression of

Table 2. Analysis of signalling pathways significantly differentially expressed ($P < 0.05$, corrected for multiple testing) between UKF-NB-4^{Hi} and UKF-NB-4^{HiGCV} cells and UKF-NB-4 in data sets of neuroblastoma patients^{27,28} PANTHER pathway analysis

Pathway	Oberthuer et al. ^{27,a} Death/relapse from disease vs no relapse rank ^c (P-value)	Asgharzadeh et al. ^{28,b} Relapse vs no relapse rank ^c (P-value)
Wnt signalling	14 (2.33×10^{-3})	1 (4.13×10^{-14})
Ras pathway	4 (6.70×10^{-7})	14 (1.58×10^{-4})
Ubiquitin proteasome pathway	44 (4.82×10^{-1})	15 (1.84×10^{-4})
Cadherin signalling pathway	76 (1.00×10^0)	23 (4.44×10^{-3})
Parkinson disease	9 (2.49×10^{-4})	27 (1.49×10^{-2})
Angiogenesis	1 (5.60×10^{-13})	2 (1.31×10^{-9})
Integrin signalling pathway	10 (2.67×10^{-4})	11 (1.63×10^{-5})
EGF receptor signalling pathway	11 (5.11×10^{-4})	6 (4.90×10^{-7})
PDGF signalling pathway	3 (4.53×10^{-8})	4 (2.74×10^{-8})
p53 pathway	5 (5.57×10^{-6})	8 (8.60×10^{-7})
Huntington disease	42 (4.34×10^{-1})	3 (2.15×10^{-8})
p53 pathways feedback loops 2	6 (6.63×10^{-6})	39 (1.18×10^{-1})
Cytoskeletal regulation by Rho GTPase	86 (1.00×10^0)	18 (4.98×10^{-4})

Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor. ^a21 out of 153 pathways were significantly differentially regulated ($P < 0.05$). ^b32 out of 153 pathways were significantly differentially regulated ($P < 0.05$). ^cPathways were ranked according to their P -values for the comparison of non-favourable vs favourable outcome with the lowest P -value being rank 1.

these genes is not commonly influenced by the long-term presence of different members of the herpes virus family (or a consequence of genetic drift). Together, these findings support the idea that the specific adaptation and selection processes during chronic HCMV infection reproducibly result in novel cell populations with characteristics clearly distinct from the parental cell lines.

Various processes might be involved in the observed establishment of a novel, more malignant neuroblastoma cell population due to long-term HCMV infection. In fully permissive normal cells such as human fibroblasts, HCMV results in cell cycle arrest and subsequent cell lysis.^{2,31} However, (at least a fraction of) chronically HCMV-infected cell cultures must escape from HCMV-induced cell cycle block and death. In this context, cell cycle deregulation is regarded as 'hallmark' of cancer,³² and HCMV may not be able to induce an effective cell cycle block in certain cancer cells.² HCMV antigen-positive glioblastoma cells were shown to divide, resulting in two HCMV antigen-expressing progeny cells.³³ In sarcoma or glioblastoma cells, virus variants arose that replicated more slowly and produced lower virus yields than the original strain used for primary infection.^{2,34,35} Therefore, changes of virus properties may contribute to the selection of a more malignant cell fraction in our model, although HCMV strain Hi91 reisolated from UKF-NB-4^{Hi} cells showed virtually the same growth kinetics after acute infection in UKF-NB-4 cells and human foreskin fibroblasts as the original virus used for primary infection.

Stress conditions activate cellular survival signalling pathways, resulting in a condition thought to favour adaptation processes in malignant cells.³⁶ In this context, HCMV is known to exert pro-inflammatory effects and to activate crucial events in survival signalling, such as NF- κ B, PI3K-Akt, MAPKs and/or JNK, and inhibit apoptosis by effects on p53 and p73, as well as by enhancing expression of anti-apoptotic proteins such as bcl-2.^{1,2,37} Moreover, HCMV is suspected to promote chromosomal instability in infected cells.^{2,38} Stable expression of the HCMV UL76 protein was sufficient to induce chromosomal aberrations in glioblastoma cells.³⁹ Indeed, the long-term presence of HCMV had resulted in chromosomal imbalances in UKF-NB-4^{Hi} cells in our experiments. As HCMV is known to also modify epigenetic features such as histone acetylation of infected cells,^{19,40–42} epigenetic mechanisms are also likely to contribute.

Finally, cancer cells are characterised by chromosomal instability, resulting in clonal evolution, that is, the permanent appearance and disappearance of novel (sub-)clones

(characterised by various mutations and/or genomic aberrations) that compete for space and resources.^{32,43} Therefore, HCMV may also exert a selection pressure on clones initially present in a cancer cell population or on clones that develop in the presence, but without the involvement, of HCMV. Five metaphases from UKF-NB-4, UKF-NB-4^{Hi} or UKF-NB-4^{HiGCV} cells were investigated by spectral karyotyping. Apparently, six abnormalities are consistently found in cells from all three cell lines, suggesting the cells to be of a common origin (Supplementary Table 6). Nevertheless, the long-term presence of HCMV may exert a selection pressure that favours the growth of (a) specific cell clone(s).

In conclusion, we present evidence that long-term HCMV infection of neuroblastoma cells can lead to the selection of a novel cell population with increased malignant properties. It is the first report demonstrating that HCMV infection can irreversibly modulate the malignant properties of cancer cells in such a way that the HCMV-induced enhanced cancer cell malignancy is retained (at least in part) even when the virus is eradicated from the cancer cells. This indicates that HCMV can act as a selection agent in cancer cells. Therefore, our findings represent a profound extension to the concept of oncomodulation^{2,3,6} and a novel way by which viruses may influence cancer diseases.

MATERIALS AND METHODS

Immunostaining for HCMV antigen expression

As described previously,⁴⁴ cells producing HCMV-specific antigens were detected by immunoperoxidase staining using monoclonal antibodies directed against the UL123-coded 72-kDa IEA1 (DuPont, Bad Homburg, Germany) or late antigen gB (kindly provided by K Radsak, Institut für Virologie, Marburg, Germany).

Virus propagation

HCMV Strain Hi91 was isolated from the urine of an AIDS patient with HCMV retinitis.²⁷ Virus stocks were prepared in human foreskin fibroblasts maintained in minimal essential medium with 4% foetal calf serum. The titres were determined by plaque titration as described previously.⁴⁴

Virus yield assay

The amount of infectious virus was determined by virus yield assay in a single-cycle assay format using human foreskin fibroblasts as described before.⁴⁴ Virus titres were expressed as 50% of tissue culture infectious dose (TCID₅₀) 120 h post infection.

Cells

UKF-NB-4 cells derived from bone marrow metastases of a patient harbouring a MYCN-amplified stage IV neuroblastoma were used.²⁶ Cells were grown at 37°C in Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated foetal calf serum and containing 100 IU/ml of penicillin and 100 µg/ml streptomycin. HUVECs were cultivated as described before.⁴⁴

Global gene expression analysis

Triplicates of UKF-NB-4, UKF-NB-4^{Hi} and UKF-NB-4^{HIGCV} cells were analysed for global cellular gene expression at the mRNA level using GeneChip HGU133 Plus 2.0 (Affymetrix) by the Fraunhofer Institut für Zelltherapie und Immunologie (Leipzig, Germany). mRNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Expression data were processed using the R/bioconductor packages 'gcrma' and 'limma' (www.r-project.org; www.bioconductor.org/) in order to detect fold changes and FDRs.⁴⁵ FDRs were corrected for multiple testing after Benjamini and Hochberg.⁴⁶ Hierarchical clustering analysis was performed and heatmaps were visualised using R (www.r-project.org).

Signal transduction pathway bioinformatics

Statistical analysis to identify significant expression changes was focusing on a pathway analysis using the PANTHER database, which identifies global patterns in expression.⁴⁷ For each expert-curated pathway in the database, potential differential expression was determined by a binomial test⁴⁸ with subsequent Bonferroni correction for multiple testing, using the PANTHER human gene reference list matching our microarrays and lists of differentially expressed genes that passed a FDR threshold of 0.05.

Array CGH

DNA was isolated from UKF-NB-4, UKF-NB-4^{Hi} and UKF-NB-4^{HIGCV} cell lines using the DNAeasy Blood & Tissue kit (Qiagen) following the manufacturer's instructions. Using DNA from cell line UKF-NB-4 as reference cell lines, UKF-NB-4^{Hi} and UKF-NB-4^{HIGCV} were profiled for DNA copy number changes by applying the Nimblegen oligonucleotide microarray platform. Hybridisations on Nimblegen HG18_WG_CGH_v2D_HX1 microarrays and microarray data processing were carried out at Source BioScience imaGenes (Berlin, Germany). The chips used contain 2.5 mio oligonucleotides tiling the human genome at an average probe spacing of 1.2 kb. Microarray images were processed in the NimbleScan 2 software (Roche NimbleGen, Madison, WI, USA).

Adhesion and transmigration assay

Neuroblastoma cell adhesion to and transmigration through HUVEC monolayers was determined as previously shown.²⁸ HUVECs were pretreated with 3-aminopropyl-triethoxy-silan (2%; Sigma-Aldrich Chemie GmbH, Munich, Germany). Neuroblastoma cells were added. After 4 h, non-adherent neuroblastoma cells were washed off. Further, cells were fixed with 1% (w/v) glutaraldehyde (Merck KGaA, Darmstadt, Germany). Adherent cells were counted in six different fields (5 × 0.25 mm²) using phase contrast microscopy. Transmigrating neuroblastoma cells were detected using a reflection interference contrast microscope with a Phloem apparatus (Nikon, Düsseldorf, Germany).

HUVEC monolayer disruption was detected and quantified, as described earlier²⁸, after 24 h of cocultivation of HUVEC monolayers with UKF-NB-4, UKF-NB-4^{Hi} or UKF-NB-4^{HIGCV} cells.

For blocking experiments, the α₅β₁ antibody VLA-5 (clone SAM-1, Hycultec GmbH, Beutelsbach, Germany) was used.

Quantitative real-time PCR

Total RNA was isolated from cell cultures using TRI reagent (Sigma-Aldrich). Quantitative real-time reverse transcriptase PCR (qRT-PCR) for viral mRNA was performed as described previously.⁴⁴ Cellular mRNA was detected using TaqMan Gene Expression Assays (Applied Biosystems, Darmstadt, Germany).

DNA was purified using the DNeasy Blood & Tissue Kit (Qiagen). Viral DNA quantification was carried out by real-time PCR as described before.⁴⁴

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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